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Enhanced Biohydrogen Production from Citrus Wastewater Using Anaerobic Sludge Pretreated by an Electroporation Technique

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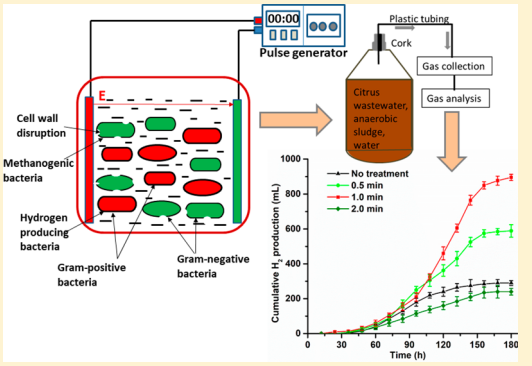
Supporting Information

ABSTRACT: In the present study, the applicability of electroporation (EP) has been investigated as a pretreatment method for enriching hydrogen producers and eliminating hydrogen consumers in anaerobic sludge (AS). Citrus wastewater was used as a feed source for biohydrogen production. Different treatment intensities (TI) of EP for 0.5 min (TI = 30 kWh/m^3), 1 min (TI = 60 kWh/m^3), and 2 min (TI = 120 kWh/m^3) were employed to observe the effects of EP on the microbial community of AS. Furthermore, sonication with a probe, sonication in a bath, and heat-shock pretreatments were also conducted to compare the hydrogen yield with EP. The cell inactivation was evaluated and visualized using colony-forming units (CFU) and field emission scanning electron microscopy (FESEM), respectively. Among the different TIs, the TI of 60 kWh/m^3 achieved higher methanogen inactivation with maximum hydrogen (896 mL) production compared to other EP pretreatments after 180 h of dark fermentation. In comparison with other pretreatments, the highest hydrogen production of 896 mL was achieved with EP treatment, followed by sonication with a probe (678 mL) and sonication in a bath (563 mL). The heat-shock pretreatment exhibited the lowest ultimate hydrogen production of 545 mL among the four different methods applied in this study. The outcome of this study suggests that EP is a promising technique for pretreating mixed cultures for the enhanced production of biohydrogen.



1. INTRODUCTION

Global warming is a key driver behind the exploration of alternative energy sources.[1,2] In this regard, hydrogen production through a biological process (biohydrogen) using the anaerobic sludge (AS) archaea can be considered to be a clean, sustainable, and environmentally friendly approach to alleviating stress on traditional fossil fuel resources.[3,4] In fermentation systems, mixed-culture microorganisms, particularly AS, are considered to be effective inocula compared to pure cultures for biohydrogen production due to advantages such as availability in nature (such as soil, wastewater, and other sources), scale-up potential, a higher tolerance of environmental fluctuations, and greater accommodative ability of the wide range of substrates.[5,6] However, AS contains various types of microbes, including hydrogen-producing and hydrogen-consuming bacteria (i.e., homoacetogens and methanogens).[7,8] Therefore, hydrogen-consuming bacteria, especially methanogens (because methanogens are dominant in AS), must be suppressed in order to enhance the performance of hydrogen production through anaerobic fermentation.[9]

A number of pretreatment methods such as heat shock (65−121 °C, 10 min−10 h),[10] acid and base,[11] aeration,[12] sonication,[13] freezing and thawing, chloroform,[14] 2-bromoethanesulfonic acid (BESA) and iodopropane,[15] ionizing irradiation,[16] load shock, and microwaves[17] have been reported to suppress methanogens in the mixed microbial community. Although heat shock is the most popular method,[18] it is not always considered to be an effective method because the activity of some non-spore forming, hydrogen-producing bacteria might be destroyed at high temperature.[19] Physical methods such as sonication and microwaves can eliminate

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methanogens but require high energy input,[20] heat generation,[21] and lower efficiency.[22] Likewise, a chemical method that employs sodium 2-BESA and iodopropane could suppress the methanogens effectively[14,15] but is less desirable for large-scale application[23] due to environmental concerns. As a consequence, these pretreatment methods have gained limited commercial application.

Recently, a microbial cell inactivation technique known as the electroporation (EP)[24] method has been used to disrupt the bacterial cell wall using high electrical pulses.[25] In the EP technique, a high-voltage pulsed electric field of direct current is delivered to the living tissues or cells for a short period of time, permeabilizing the cell membrane for transfection and transformation.[26] EP is able to create an electrical charge on the dielectric microbial cell wall and plasma membrane, which causes the irretrievable breakdown of the microbial cell.[27] It is postulated that a certain EP treatment intensity could inactivate the methanogenic archaea of AS. In particular, the sensitivity of EP treatment depends on the shape, size, and types of the microbes as well as the thickness of the cell wall. For example, Gram-positive bacteria (mostly hydrogen-producing) are more resistant to the electric field compared to Gram-negative bacteria (mostly hydrogen-consuming).[28] Similar findings were reported by Mazurek et al.,[29] who stated that the Gram-negative bacteria are more sensitive to EP treatment than Gram-positive bacteria. Stiffer and thicker cell walls of Gram-positive bacteria have the potential to create protection against a pulsed electrical field.[29] In another study, Pothakamury et al.[30] observed that the inactivation time was varied for Gram-positive and Gram-negative species when EP was applied to the bacteria. Nevertheless, EP needs only a few minutes to inactivate methanogens depending on the treatment intensity, while the treatment time was 10 min to 3 days for other pretreatments.[10] Therefore, the EP technique could effectively be applied to eliminate methanogenic bacteria from an anaerobic sludge mixed-culture consortium, which has not been addressed in the literature.

Consequently, in this study, we report for the first time the effects of EP as a pretreatment technique for enrich hydrogen-producing bacteria by inactivating the methanogens from AS. The efficiency of EP was evaluated through the determination of the cell inactivation rate and also the measurement of cumulative hydrogen production.

2. MATERIALS AND METHODS

2.1. Design of an EP Circuit and Reactor Fabrication. The design of an EP circuit (comprising a high voltage power supply, a pulse generator, and a switching circuit) and the fabrication of a reactor have been described by Karim et al.[24] In brief, plexiglass was used to prepare the EP reactor with dimensions of 5.0 cm × 2.5 cm × 5.0 cm. Two round-shaped stainless-steel plates with a surface area of 10.2 cm^2 were used as electrodes in the reactor. The distance between two electrodes was maintained at 2 cm.

2.2. Source of Microorganism and Inoculum Preparation. AS was collected from an anaerobic digester of a local palm oil mill located in Gambang, Pahang, Malaysia (latitude 3.709472, longitude 103.102686). The samples were filtered with a Whatman no. 1 filter paper to eliminate submerged solids and debris and subsequently preserved at 4 °C to prevent further alteration. The sludge suspension was serially diluted (10^{-1} to 10^{-6}), and the enrichment of the culture was carried out by preparing an overnight culture in LB (Luria–Bertani) broth (incubated at 37 °C with a shaking speed of 150 rpm).

2.3. Sample Collection. The raw citrus wastewater (CW) was obtained from a food company located in Johor Bahru, Malaysia. The samples were kept at 4 °C to avoid further deterioration by indigenous microbial activity. Thereafter, the submerged solids and debris were removed using a Whatman no.1 filter paper and subsequently sterilized by autoclaving at 121 °C for 15 min. The 50% diluted CW (raw CW/deionized water ratio = 1) was used as a substrate to enriching bacteria. The pH was adjusted to 6.0 ± 0.1 by adding 1 N NaOH.

2.4. Seed Sludge and Pretreatment. Four different pretreatment methods (EP, heat shock, sonication with a probe, and sonication in a bath) were used in this study. The initial cell concentration was adjusted to 2×10^6 CFU/mL, and it was maintained using the optical density (OD) at 600 nm (Shimadzu, model UV-180 240 V) for each batch. Prior to the EP treatment, the EP reactor was cleaned and sterilized by flushing with 70% ethanol solution followed by sterilized deionized water. The conductivity of the culture medium was 90 μS/m. High voltage (4 kV) with a pulse frequency of 100 Hz was applied to the reactor. It should be noted that all of the experiments were performed in triplicate to confirm the observation of each treatment. The treatment intensity (TI, kWh/m^3)[31] was calculated using eq 1

$$TI = K\frac{V^2 D f \sigma t}{L^2} \tag{1}$$

where V is the applied voltage (kg $m^2/C\ s^2$), D is the pulse width (s/pulse), f is the pulse frequency (pulse/s), σ is the sample conductivity in S/m (s C^2/ kg m^3), L is the distance between electrodes (m), t is the treatment time in the treatment chamber (s), and K is the constant for unit conversion (2.778×10^{-7} kWh/J).

The bath sonication pretreatment was performed in an ultrasonic bath at a frequency of 35 kHz and a power of 240 W at room temperature (27 ± 2 °C) for 40 min. For the sonication treatment with a probe, a laboratory-scale ultrasonic probe (Qsonic, LLC; model Q500) was used at a frequency of 20 kHz with a power of 500 W for 20 min. The inoculum was treated without temperature control, and sonication pulses were set to 5 s on and 5 s off. The heat-shock pretreatment was performed in a water bath maintained at a temperature at 100 °C for 60 min to inhibit the activity of methane-producing bacteria. The heat-pretreated sludge was then used as an inoculum for fermentative hydrogen production.

2.5. Cell Inactivation Rate. The spread plate counting technique was employed to determine the cell viability for the untreated and EP-treated samples. Diluted cell suspensions (10^{-6}) of each sample were evenly spread over the agar plate. Thereafter, bacterial colonies were counted on suitable agar plates after incubation (24 h), and the count was expressed as colony-forming units (CFU) per milliliter of sample as in eq 2

$$\text{cell inactivation rate} = \frac{(\text{initial CFU at } t_i - \text{final CFU at } t_n)}{\text{initial CFU at } t_i} \times 100 \tag{2}$$

where t_i is the number of initial CFU (before treatment, i = 0 min) and t_n is the number of CFU at a different treatment time (after treatment, n = 0.5, 1, 2, and 3 min).

2.6. FESEM Analysis. The surface morphology of bacterial cells was analyzed using field emission scanning electron microscopy (FESEM, JEOL, JSM-7800F, Japan) at a voltage of

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5 kV. The samples were collected from the EP reactor before and after (at different time intervals) treatment and then taken on selective slides. Thereafter, all samples were coated with platinum using an ion-sputtering system. Finally, the cells were visualized under the microscope.

2.7. Molecular Analysis of the Microorganism. Biolog GEN III analysis for untreated and EP-treated samples was performed to identify the microorganisms. The molecular characterization of microorganisms was performed as presented in the Supporting Information (Figures S1 and S2).

2.8. Bioreactor Design and Fabrication. Four sets of bioreactors were fabricated to study the effects of different EP treatment on AS in producing biohydrogen such as the control (without EP treatment), 0.5 min of EP treatment (TI = 30 kWh/m^3), 1 min of EP treatment (TI = 60 kWh/m^3), and 2 min of EP treatment (TI = 120 kWh/m^3). The 1 L Scott bottle was used as a bioreactor to produce hydrogen. It was sealed with silicone-type rubber stoppers. The whole experiment was operated in batch mode. In each reactor, 100 mL of culture was used to inoculate 700 mL of citrus wastewater (CW). The reactors were then sealed with sterilized screw caps. Thereafter, the headspace of all reactors was purged with nitrogen gas to create an anaerobic environment. The anaerobic dark environment under mesophilic conditions (36 °C) was maintained throughout the experiment (180 h). The gas produced from the reactor was collected and analyzed every12 h. The reactors were placed in a reciprocal shaker (120 rpm) and maintained at a constant temperature of 36 °C during the fermentation process. The aforementioned procedures were repeated for sonication in a bath, sonication with a probe, and the heat-shock-pretreated inoculum. All of the experiments were replicated three times.

2.9. Analytical Methods. The volume of biogas produced in the bioreactors was measured by a water displacement technique. The hydrogen content of biogas was determined in a gas chromatograph (Agilent 6890 series GC system) equipped with an HP-PLOT Q capillary column (30 m × 0.53 mm × 40 μm) and installed with both thermal conductivity (TCD) and flame ionization (FID) detectors. In addition, nitrogen gas was used as the carrier gas at a flow rate of 45 mL/min. The temperatures of the column, injector, and detector were 40, 100, and 150 °C, respectively.

2.10. Wastewater Characterization. The biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS), and pH were determined according to the standard methods described in APHA.[32,33] Soluble carbohydrates were determined according to the procedure described by Johnson and Sieburth with minor modifications.[34] In brief, 0.05 mL of freshly prepared sodium borohydride solution (14 mg/L) was added to 1 mL of a filtered CW sample to reduce the sugars to the corresponding alcohols, and the sample was incubated overnight at ambient temperature. After the addition of 0.36 mol/L HCl, 0.025 mol/L periodic acid solution, 0.25 mol/L sodium arsenite solution, and 2 mol/L HCI at various stages, the amber color developed and disappeared rapidly. Then, 0.2 mL of 3-methyl-2-benzothiazolinone hydrazone reagent was added, and the solution was heated in a boiling water bath for 3 min. After cooling, 0.2 mL of 5% iron(III) chloride solution was added, and the solution was held at 25 °C for 20 min. After color development, 1 mL of acetone was added, and the absorbance was recorded immediately at 635 nm using a double-beam UV−visible spectrophotometer (Shimadzu, model UV- 180 240 V). The soluble carbohydrate concentration in the sample was determined from the calibration curve. The calibration curve was prepared with different known concentrations of standard glucose solutions. The characteristics of raw CW are presented in Table 1.

Table 1. Characteristics of Raw CW

characteristics	value
pH	4.17
soluble carbohydrates, g/L	36.67
TSS, g/L	12.23
VSS, g/L	8.87
BOD, g/L	35.56
COD, g/L	53.87

3. RESULTS AND DISCUSSION

3.1. Cell Inactivation by EP Treatment. The effect of EP treatment on the bacterial cells is demonstrated in Figure 1. It

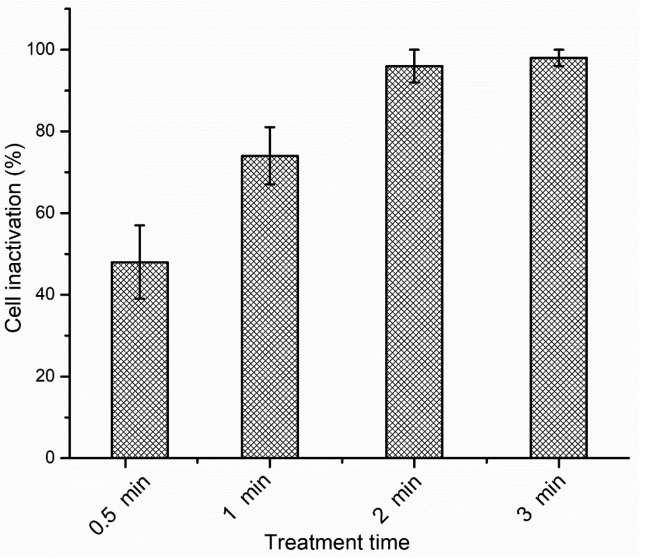

Figure 1. Effect of EP treatment time on bacterial cell inactivation (where the voltage, frequency and distance between electrodes were 4 kV, 100 Hz, and 2 cm, respectively).

can be seen that the cell inactivation was gradually increased to up to 2 min of treatment time, where 96% of cell inactivation was achieved. Thereafter, the longer treatment time was immaterial but energy-consuming because cell inactivation reached a plateau and no significant changes were observed. The higher cell inactivation might be attributed to the cell wall damage of bacteria, leading to the increase in the number of dead cells in the EP reactor.

The inactivation of pure cultures by applying different electric fields has been demonstrated in several publications.[35−39] In a study, the *Escherichia coli* populations were quickly reduced with the application of EP at initial pulses (12.34 kV/cm and 2.7 pulses), and then a minor effect was observed on the microbial cell reduction with the subsequent pulses (30 kV/cm and 30 pulses).[35,36] A similar trend was recorded in the present study, where 48% of the anaerobes were rapidly inactivated within the first 30 s. Spilimbergo et al.[38] stated that the viability of bacteria (*E. coli* and

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Staphylococcus aureus) might be lessened with the increase in the number of pulses and electric field intensity. However, the inactivation of anaerobes by EP from mixed cultures has not been reported so far. Therefore, it is assumed that a certain range of EP treatment could be a promising technique for inactivating most of the microbes of AS.

3.2. Cell Disruption Visualization. The effect of EP on the morphology of bacterial cells was visualized by FESEM and is presented in Figure 2. Heterogeneous populations of cells

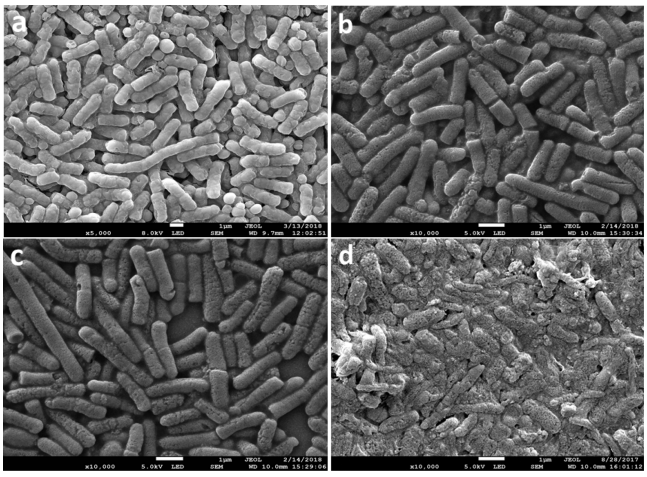

Figure 2. FESEM image of microbial communities of anaerobic sludge for (a) untreated samples and samples treated with EP for (b) 0.5, (c) 1, and (d) 2 min.

with smooth surfaces were observed in an untreated sample (Figure 2a). On the other hand, some cells with a damaged surface and some others with a smooth surface were observed in Figure 2b,c. However, most of the cells with extensively damaged surfaces were found after 2 min of EP treatment (TI = 120 kWh/m^3), as presented in Figure 2d. The greater number of damaged cells achieved after the 2 min treatment compared to the 0.5 min treatment (TI = 30 kWh/m^3) might be due to the higher TI of EP.

It can be seen from the FESEM images that EP damaged the microbial cells by creating cleavages on the surface of the cells. Generally, peptidoglycan is an essential structural component of the bacterial cell wall, and its architecture in the cell wall network contributes to maintaining the shape and protecting the cell from bursting.[40] The peptidoglycan network is organized in cables perpendicular to the long axis of the cell for vegetative bacteria.[40] This cable network might be disorganized by the application of an EP treatment. In our previous study,[41] it was observed that the higher treatment intensity induced irreversible permeabilization of the cell wall, leading to its disruption. Moreover, EP has an intense effect on cell viability and changes in the cell wall structure, resulting in increased wall porosity.[42] Dutreux et al.[43] reported that the cell wall of *E. coli* could be ruptured by EP. It was observed by Loghavi et al.[44] that EP can trigger pore formation on the cell wall when the electric charge exceeds the critical rupturing value. However, when the cells are exposed to an external electric field of sufficient amplitude and duration, the electrical conductivity and permeability of the cell might be increased significantly and thus pores could be induced in the cell wall, where they are increased in size and number with pulse duration.[45] Therefore, such a disruption to the cell wall during EP might have damaging effects on the viability of cells immediately after treatment.

Biolog GEN III analysis for untreated and 1 min EP-treated (TI = 120 kWh/m^3) bacterial samples was performed to confirm the inactivation of methane-producing bacteria. The predominant microorganisms from the untreated and pretreated inocula were isolated and characterized as presented in Figures S1 and S2. A wide range of microbial communities such as *Rhodocyclaceae*, *Acetobacteraceae*, *Solimonadaceae*, *Rodopseudomonas*, *Bacillus*, and *Methanobacteriales* (archea) were found in both inocula. However, it is important to note that although the methanogenic archea were found in both communities, some predominant species such as *Methanothermous fervidus*, *Methanocullous thermophilies*, and *Methanobacillus arboreus* were not observed in the EP-treated inoculum (Figure S2). The result suggests that EP might have inhibited the predominant methanogenic archea in AS, which would have decreased the methane formation, resulting in an enhancement in biohydrogen production. However, further studies are needed to understand the effect of EP on different microbial communities and the mechanisms involved to effectively inactivate the methanogens in order to improve the performance of biohydrogen producers in mixed cultures.

3.3. Biohydrogen Production. The total gas accumulated during the 180 h fermentation of CW with different EP pretreated inocula and without pretreatment is presented in Figure 3. It can be observed that the highest amount of gas

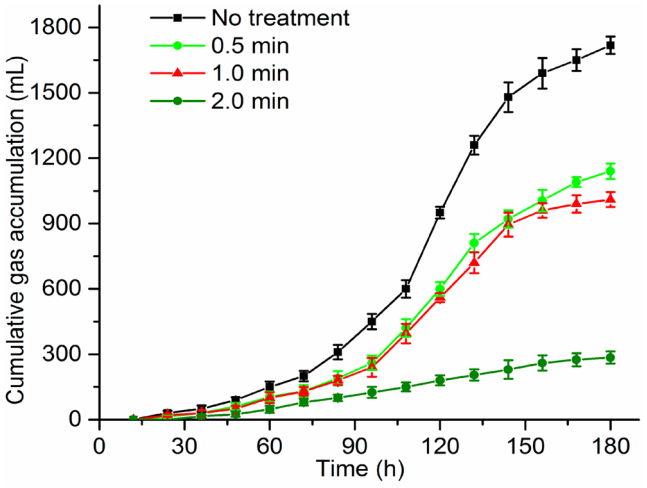

Figure 3. Cumulative gas accumulation during the 180 h of fermentation with different EP-pretreated inocula.

(1718 mL) was obtained for the reactor with the inoculum without pretreatment, whereas 1140, 1010, and 285 mL of gas were accumulated for 0.5, 1, and 2 min EP-pretreated inocula, respectively. Figure 4 shows the time course of cumulative hydrogen production during the 180 h fermentation of CW. It was observed that after 180 h of fermentation the 0.5 min EP-pretreated inoculum produced 589 mL (52% v/v) of hydrogen, 1 min pretreated inoculum produced 896 mL (88.7% v/v) of hydrogen, and untreated inoculum produced 290 mL (17% v/v) of hydrogen. However, the 2 min EP-treated inoculum produced only 240 mL (84% v/v) of hydrogen. It should be noted that during the first 36 h the amount of hydrogen production was not increased significantly for all inocula, which might be due to the adaptation and

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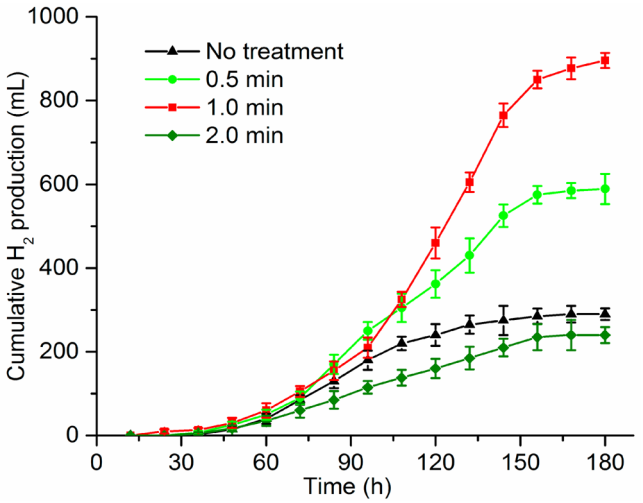

Figure 4. Performance of cumulative hydrogen production during the 180 h fermentation with different EP-pretreated inocula.

concentration of hydrogen-producing bacteria in the CW medium.[46] Thereafter, the production of hydrogen was increased gradually over the 36−180 h period of the fermentation, and then a stabilization in yield was found for all of the samples. This could be attributed to the complete degradation of the substrates.[47,48]

The maximum hydrogen production was observed in the 1 min EP-pretreated inoculum, which suggests that a greater number of hydrogen-producing bacteria have survived while hydrogen consumers were inactivated. In contrast, the total gas production is the highest (Figure 3) for the untreated bacteria seed than for all other pretreated conditions because a greater number of cells and diverse types of microorganisms were present in untreated AS.[49] Although the total volume of gas was higher, the hydrogen production was comparatively lower in the untreated inoculum. This might be attributed to the fact that the methanogens and hydrogen consumers of AS have suppressed the activity of hydrogen producers because methanogens are dominant in AS.[50] However, the lowest total gas accumulation (Figure 3) and hydrogen production (Figure 4) were observed in the 2 min EP-pretreated inoculum, which could be due to the inactivation of most of the cells and a substantial decrease in bacterial diversity, predominantly in the methanogens and some of the hydrogen producers as well.[51] Although the production of hydrogen was lower for the 0.5 min treated inoculum than for the 1 min EP-treated inoculum, a comparatively higher amount of cumulative gas production was observed under these conditions. This could be attributed to the survival of a substantial number of methanogens during the lower treatment intensity of EP.

Typically, the pretreatment of mixed cultures before the inoculation of fermentation media performed better in producing hydrogen than did the untreated seed cultures. Hydrogen-producing bacteria have the ability to form spores, and this characteristic gives them a better chance of surviving than some non-spore-forming hydrogen-consuming bacteria.[11] The characterization analysis of anaerobic consortia by Maintinguer et al.[52] showed that the predominance of Gram-positive rods and rods with end-spores in anaerobic consortia has a strong influence on hydrogen production. Thus, it is assumed that a certain range of EP treatment intensity could inactivate the Gram-negative methane-producing bacteria. It was revealed that the 1 min EP treatment is able to inactivate the maximum non-spore-forming bacteria, thus producing more hydrogen. Spore-forming hydrogen-producing bacteria such as *Bacillus* and *Clostridium* are able to form protective spores having a resistance to high electric pulses. It has been identified that spores of *Bacillus cereus* and *Bacillus polymyxa* are impervious to electric field strengths of up to 30 kV/cm.[53] It is generally understood that the high forces associated with the pulsed electric field can damage the cell wall of methanogens because the composition of the cell wall of methanogens is fundamentally different from that of the nonmethanogens' bacterial cell wall, and the methanogens are phylogenetically and physiologically distinct from all other cell types.[54] Moreover, the phospholipid membrane could be damaged during the application of high-voltage electric pulses. One recent study demonstrated that up to 14% of the membrane surface could be lost upon electric field application of 2 min with a field intensity of 5.7 kV/cm.[55] Thus, the certain range of EP treatment intensity would degrade the phospholipids and peptidoglycan of methanogenic archaea and lead to the disruption of cells. Therefore, it is demonstrated that the mixed cultures pretreated with EP at a treatment intensity of 60 kWh/m^3 (1 min of EP) might be suppressed methanogens as well as most of the other hydrogen-consuming bacteria.

The cumulative hydrogen production for four different pretreatment methods and the untreated AS after a duration of 180 h of fermentation is presented in Figure 5. As shown in

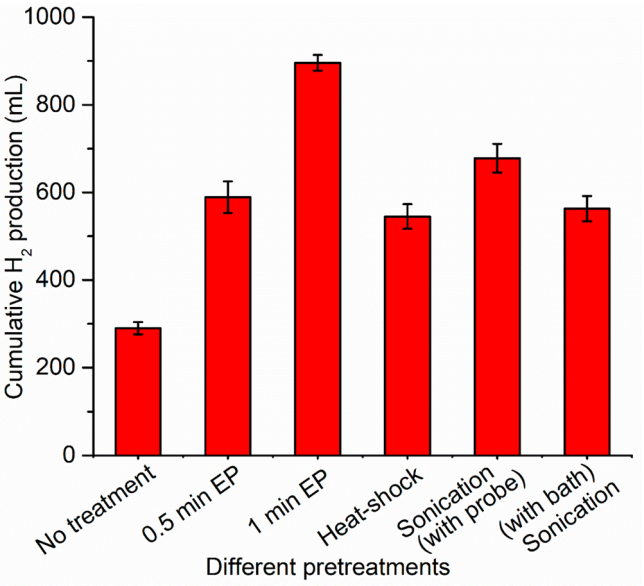

Figure 5. Cumulative hydrogen production after the 180 h fermentation of citrus wastewater with different pretreatment methods.

Figure 5, the ultimate hydrogen production of pretreated sludges was higher than that of untreated sludge (290 mL). The highest hydrogen production of 896 mL was achieved with 1 min of EP treatment, followed by 20 min of sonication with probe (678 mL), 0.5 min of EP treatment (589 mL), and 40 min of sonication in a bath (563 mL). However, the heat-shock pretreatment exhibited the lowest ultimate hydrogen production of 545 mL among the four different methods applied in this study.

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Table 2. Comparison of Different Pretreatment Methods of AS in Terms of the Hydrogen Yield in Batch Studies

treatment method	treating condition	substrate	hydrogen yield mol H_2/mol substrate		increase in hydrogen yield, %
			control (without pretreatment)	after pretreatment	
acid[15]	pH 3.0 adjusted with OPA, 24 h[c]	dairy wastewater (10.4 g/L COD)	0.0018[a]	0.0079[a]	338
base[19]	pH 10.0 adjusted with 1 mol/L of NaOH, 24 h	glucose	0.29	0.56	93
chemical[56]	10 mmol BESA mixed (at 200 rpm) for 30 min[b]	sucrose	0.30	1.01	237
ultrasonication (with temperature control)[13]	power: 500 W, frequency: 20 kHz, duty cycle: 50%, 20 min, 30 °C	glucose	0.70	1.55	121
ultrasonication (without temperature control)[13]	ultrasonic probe (model VC-500, 500 W, and 20 kHz); duty cycle: 50%	glucose	0.70	1.03	47
heat shock[16]	100 °C for 15 min	glucose	0.80	1.59	99
ionizing irradiation[16]	5 kGy dose of γ irradiation; irradiated at a dose rate of 286 Gy/min ; 25 °C	glucose	0.80	2.35	194
aeration[22]	controlling the DO (<0.5 mg/L) and feeding glucose for 12 h; 25 °C	glucose	1.45	1.80	24
microwave[57]	power output of 860 W and the frequency of 2450 MHz, 2 min	glucose	1.45	1.92	32
pH + heat + chemical[15]	100 °C, pH 3.0 adjusted with OPA[c] 24 h + 0.2 g/L BESA[b] under an anaerobic environment for 24 h	dairy wastewater (10.4 g/L COD)	0.0018[a]	0.0108[a]	500
electroporation (present study)	4 kV, 100 Hz, 0.5 min	CW (25.5 g/L COD)	0.54[a]	1.45[a]	169
electroporation (present study)	4 kV, 100 Hz, 1 min	CW (25.5 g/L COD)	0.54[a]	2.24[a]	315

[a]The hydrogen yield was calculated as millimoles of hydrogen produced per gram of COD influent. [b]BESA: 2-bromoethanesulfonic acid. [c]OPA: concentrated *ortho*-phosphoric acid.

The hydrogen yield obtained by applying different pretreatment methods has been compared with the present study as shown in Table 2. The results of this study showed a drastic increase (315%) in the hydrogen yield using 1 min of EP treatment (TI = 60 kWh/m^3) of AS. Compared to other physical treatments such as ultrasonication, heat shock, aeration, microwaves, and ionizing irradiation, EP had a higher efficiency in enriching the hydrogen-producing bacteria, leading to a higher hydrogen yield. The higher hydrogen yield is crucial to large-scale biohydrogen production. Nevertheless, the scale-up process of fermentative biohydrogen production is challenging because of the unavoidable methanogenic activity of mixed cultures in the system. The methanogenic activity in the system leads to the alternative utilization of substrates rather than hydrogen production. It has already been demonstrated in the present study that a certain range of EP treatment can inactivate most of the methanogens of AS. Furthermore, a standard continuous EP treatment process can be developed with a pulse generator that enables continuous pulse treatment, flow chambers with properly designed electrodes, and a precise fluid handling system in order to facilitate EP pretreatment on a large scale. Therefore, the EP technique could potentially be applied in the industrial system to inhibit methanogens in mixed cultures and prevent alternative routes of substrate utilizations. However, further detailed studies are needed to elucidate the feasibility of EP in industrial applications.

4. CONCLUSIONS

The overall findings of this study suggest that EP is a very promising pretreatment method for enriching hydrogen production by using AS seeds in dark fermentation. Among the different TIs of EP, the moderate intensity (TI = 60 kWh/m^3) had the greatest ability to enrich the hydrogen-producing microbes on the AS, resulting in an enhancement of biohydrogen production in the reactor. The highest cell inactivation (96%) was achieved by TI = 120 kWh/m^3. However, the maximum cumulative hydrogen yield was achieved with a TI of 60 kWh/m^3, where 74% cell inactivation was observed. The untreated seeds produced 290 mL of cumulative hydrogen after 180 h, which was 3 times lower than for the EP-treated (TI = 60 kWh/m^3) seeds. Furthermore, the EP technique has greater potential compared to heat shock, sonication with a probe, and sonication in a bath for enriching the hydrogen producing archaea from the AS in order to enhance biohydrogen production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.iecr.8b03586.

Phylogenetic tree analysis of anaerobic sludge before EP treatment and phylogenetic tree analysis of anaerobic sludge after EP treatment (PDF)

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Notes

The authors declare no competing financial interest.

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